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METABOLIC ACTIVITIES AND CLEAVAGE OF EGGS OF THE SEA URCHIN, ARBACIA PUNCTULATA
A REVIEW, 1932–1949 [1]

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CONTENTS

[1] This review was prepared at the invitation of the Editorial Board.

A. Introduction

The literature on the chemical and physical properties of eggs of *Arbacia punctulata* was summarized by Harvey (1932). During the ensuing period, the use of eggs of Arbacia and other sea urchins as representative cells for the study of the fundamental properties of animal tissue has greatly increased.

Two problems, closely related to each other, have been extensively studied: the analysis of the chemical events associated with fertilization; and the elucidation of the chemical processes by which energy liberated by oxidation of foodstuffs is made available for mitotic processes and cleavage.

The first of these two problems is the subject of excellent reviews by Tyler (1948) and Runnström (1949) and will therefore be dealt with here only incidentally.

The second problem is constantly gaining in interest because of its fundamental relationship to normal and pathological growth processes. As the result of investigations by both American and European workers there is probably no cell or tissue, with the possible exception of skeletal muscle, for which information relating enzymatic processes to function is as complete as it is for the sea urchin egg.

The principal objective of this paper is to bring together what is known about the relation of metabolic enzymes and metabolic processes to cleavage in the Arbacia egg. In addition, because of their potential bearing on this objective, the effects of chemical agents upon metabolic activity and cleavage are recorded; those agents which have known effects upon enzymes are stressed, but other agents are included. Chief attention will be given to the years 1932–1949 inclusive, publications prior to 1932 having been covered in the aforementioned review by Harvey (1932). Observations upon other marine eggs will be introduced where a comparison with the results on Arbacia is desirable.

Every effort has been made to leave no significant aspect of the relation of metabolic processes to cleavage unmentioned, but the bibliography does not include every publication in this field, as a given set of observations has in many instances been published in more than one place.

B. Oxygen Consumption

1. Units. Oxygen uptake of marine eggs has usually been expressed as c. mm. per 10^6 eggs or as c. mm. per hour per 10 c. mm. eggs. To permit expression of results in various units, the following data are useful.

Egg dimensions, average diameter, 74 μ; surface, 17,200 μ^2; volume, 212,000 μ^3 (Harvey, 1932). By diffractometer method, the mean volume is 228,000 μ^3 (Korr, 1937).

Density, 1.09 (Harvey, 1932).

Dry weight, 24.2 per cent of wet weight (Ballentine, 1940a); 23.9 per cent (Hutchens et al., 1942).

Nitrogen content, 0.107 mg. nitrogen per mg. dry weight, 5.86 mg. nitrogen per 10^6 cells (Ballentine, 1940a); 0.101 mg. nitrogen per mg. dry weight, 5.93 mg. nitrogen per 10^6 cells (Hutchens et al., 1942). Egg protein is assumed to be 6.25 times the egg nitrogen content.

Cells per mg. dry weight, 17,600.

Cells per 10 c. mm., 46,500.

Cells per wet gram, 4,300,000.

Weight of 10 c. mm. eggs, 10.9 mg. wet, 2.63 mg. dry.

2. Determination of Egg Volume. Measurements of egg volume have been commonly made by the hematocrit method. The hematocrit values obtained depend upon the period and intensity of centrifugation, and upon whether the volume which is measured is the total volume (eggs plus sedimentable jelly) or egg volume only. The most frequently used hematocrit value is that for the eggs only, as determined by centrifuging 5 minutes or more at 2000 × gravity or above, i.e. to an essentially constant volume. The egg volume as thus determined by hematocrit is about 8 per cent larger than calculated from hemocytometer measurements (Shapiro, 1935; Clowes and Krahl, 1936). The volume of a given number of eggs is 8 per cent smaller after fertilization than before (Glaser, 1924).

When results are expressed in terms of egg volume, it is helpful to state whether volume is determined on fertilized or unfertilized eggs.

3. Oxygen Consumption of Whole Eggs. Measurements on oxygen consumption of whole eggs are recorded in Table I.

The rate of oxygen consumption is strongly dependent upon the degree of trauma to which the Arbacia eggs are subjected prior to or during the measurement of oxygen consumption. Shaking rates above 48 per minute at an amplitude of 7.5 cm. (Whitaker, 1933a), light centrifuging during washing of eggs (Velick, 1941) or any other treatment which tends to break down the egg surface layers produces an increase in rate of oxygen consumption. Tyler, Ricci, and Horowitz (1938) found that the rise in oxygen consumption of unfertilized eggs, which begins some five hours after the eggs are shed, does not occur when the bacterial population in the sea water around the eggs is not allowed to increase.

For these reasons the absolute value of the oxygen consumption of *Arbacia punctulata* eggs cannot be specified except in relation to the conditions used for measurement. It follows that measurements designed to show effects of chemical agents and other treatments should be made under very carefully controlled and precisely described basal conditions. When the experimental conditions are kept constant, the values are reproducible from season to season.

Increase in oxygen consumption upon fertilization. The change in oxygen consumption of *Arbacia punctulata* eggs upon fertilization has been repeatedly studied since Warburg (1908) first showed oxygen consumption of the *Arbacia pustulosa* egg to be increased upon fertilization (Loeb and Wasteneys, 1911; Tang and Gerard, 1932; Whitaker, 1933a; Rubenstein and Gerard, 1934). The last named authors found that the factor by which oxygen consumption increases upon fertilization is a variable, its magnitude depending upon temperature. At 11° C., the fer-

TABLE I

Oxygen consumption of unfertilized and of fertilized eggs of Arbacia punctulata in natural sea water. The measurements are tabulated in chronological order. Where conversion factors were necessary, those given earlier in this paper were applied to the original measurements. A discussion of the validity of the numerical values is given in the text.

Observer	T° C.	Fert. (F) or unfert. (U)	Period of measurement, time after fertilization	Oxygen consumption
			hours	c. mm. per hr. per 10 c. mm. eggs
Tang (1931)	24.7	U	—	1.6 [1,2]
		F	0.25–1	7.9
Tang and Gerard (1932)	25	F	0.25–1	5.5 [1]
Whitaker (1933a, 1935)	21	U	—	0.4–0.5
		F	0.38–0.63	2.0
Gerard and Rubenstein (1934)	21	U [3]	—	0.9
Shapiro (1935a)	25.9	U	—	1.5
		F	—	3.9
Clowes and Krahl (1936)	20	U	—	0.6–0.7
	12	F	0.42–3	1.7
	20	F	0.42–3	3.1
	27	F	0.42–3	5.5
Korr (1937) [4]	15	U	—	0.2
	20		—	0.5
	25		—	1.0
	12	F	—	1.7
	15		—	2.1
	20		—	3.0
	25		—	4.7
Tyler, Ricci, and Horowitz (1938)	20	U	—	0.7
Krahl and Clowes (1938a)	20	F	0.42–3	3.1
Hutchens, Keltch, Krahl, and Clowes (1942)	20	F	1–4	2.4
			4–7	4.4
			7–11	4.9
			11–16	5.9
			16–26	6.8
Keltch and Clowes (1947)	20	F [5]	—	4.8

[1] The eggs were centrifuged lightly during washing, which tends to raise the oxygen consumption.

[2] Values are also given for various partial pressures of oxygen.

[3] Data are also given for change in oxygen consumption upon fertilization at temperatures from 11–29.9° C.

[4] The effects of pyocyanine, cyanide, and combinations of the two were also measured over the temperature range 11–26° C.

[5] Artificial parthenogenesis by hypertonic NaCl; 98 per cent membrane formation, 18–45 per cent cleavage.

tilized rate is about ten times the unfertilized; at 29.9° C., it is only twice that of the unfertilized.

At 20° C., the increase in oxygen consumption of Arbacia eggs is as large after artificial parthenogenesis (hypertonic NaCl) as after sperm fertilization (Keltch and Clowes, 1947). Mechanisms by which the increase in oxygen consumption upon fertilization may be brought about have been discussed by Whitaker (1933b), Krahl, Keltch, Neubeck and Clowes (1941), Goldinger and Barron (1946), Runnström (1949), Korr (1937), Ballentine (1940), and others. Whitaker (1933b) has summarized the measurements upon oxygen consumption of eggs of other marine forms: *Asterias forbesii, Cumingia tellinoides, Chaetopterus, Fucus vesiculosus, Nereis limbata.*

Oxygen consumption of the Arbacia egg increases gradually after fertilization until the egg is hatched, and then more markedly (Hutchens *et al.*, 1942). Similar studies on fertilized eggs of European sea urchins have been made by Gray (1927), Lindahl and Holter (1941), and Borei (1948). Zeuthen (1947) reported that, during the 5th to 8th cleavages of Psammechinus, the oxygen consumption is higher in the prophase, and lower in the later phases of a given mitotic cycle.

4. Oxygen Consumption of Egg Fragments. By high speed centrifuging in solutions of suitable specific gravity, unfertilized Arbacia eggs may be broken into a light half, containing the nucleus, the mitochondria, and some yolk; and a heavy half, containing the pigment granules and the major portion of the yolk. It has been found that the oxidative activity of the heavy half is somewhat greater than that of the light half, whether measured by oxygen consumption (Shapiro, 1935) or as reducing activity toward ferricyanide (Ballentine, 1940c). The combined oxygen uptake of the two halves of the unfertilized egg is 29 per cent greater than that of the whole egg, when proper allowance was made for volumes of the fragments; the sum of the respiratory rates of the two kinds of fertilized halves is 17 per cent less than that of the whole fertilized egg. Addition of p-phenylenediamine to the light halves produces a relatively smaller increase in oxygen consumption than addition of the same agent to the heavy halves (Boell *et al.*, 1940).

5. Respiratory Quotient. The respiratory quotient of unfertilized Arbacia eggs has apparently not been measured. That of the fertilized eggs appears to vary with the batch of eggs used; the rate of glycogen disappearance is low when the respiratory quotient is low. Observed R.Q. values are, for various periods after fertilization (Hutchens, Keltch, Krahl, Clowes, 1942): 1–4 hours, 0.85 (0.76–0.94); 1–7 hours, 0.88 (0.75–1.00); 1–11 hours, 0.90 (0.75–1.04); 1–16 hours, 0.88 (0.79–0.97); 1–25 hours, 0.86 (0.84–0.98). The respiratory quotient of the *Arbacia pustulosa* egg was found to be: unfertilized, 1.06; fertilized, 0.95 (Ashbel, 1930).

6. Oxygen Consumption of Sperm. See Barron, Seegmiller, Mendes, and Narahara (1948); Benedict and Barron (1946).

C. Rate of Cleavage

The times from fertilization to 50 per cent cleavage at 20° C. were observed by Fry (1936) to be: first cleavage, 69 minutes; second cleavage, 107; third cleav-

age, 145. For times to first cleavage at other temperatures, and times to other stages of development, see Harvey (1932).

Effects of various physical or chemical agents upon cleavage rate have been measured by two methods. In one, employed first by Smith and Clowes (1924), and used by Clowes, Krahl, and coworkers for their measurements, the control and experimental aliquots of eggs are allowed to develop until the control eggs have undergone several divisions; the eggs are then fixed in 0.1 per cent formaldehyde, and the percentage of the total in each state of division is determined by counting. The extent of division is expressed numerically according to the number of divisions undergone: no division, 0; 2 cell, 1; 4 cell, 2; 8 cell, 3; 16 cell, 4. For example, a population 25 per cent 4 cells, 70 per cent 8 cells, and 5 per cent undivided would have undergone, on the average, $0.25 \times 2 + 0.70 \times 3 + 0.05 \times 0 = 2.6$ divisions per egg. Cleavage in presence of various agents can be expressed as a fraction of control rate by dividing the divisions per egg in the experimental sample by the divisions per egg in the control.

In the second method of determining cleavage rate, the time to reach 50 per cent first cleavage is determined by serial fixation and counting of samples. The effect of experimental conditions can then be expressed as minutes of cleavage delay (or acceleration), as ratio of time required for the experimental sample to that required for control, or as per cent delay or acceleration relative to the control rate.

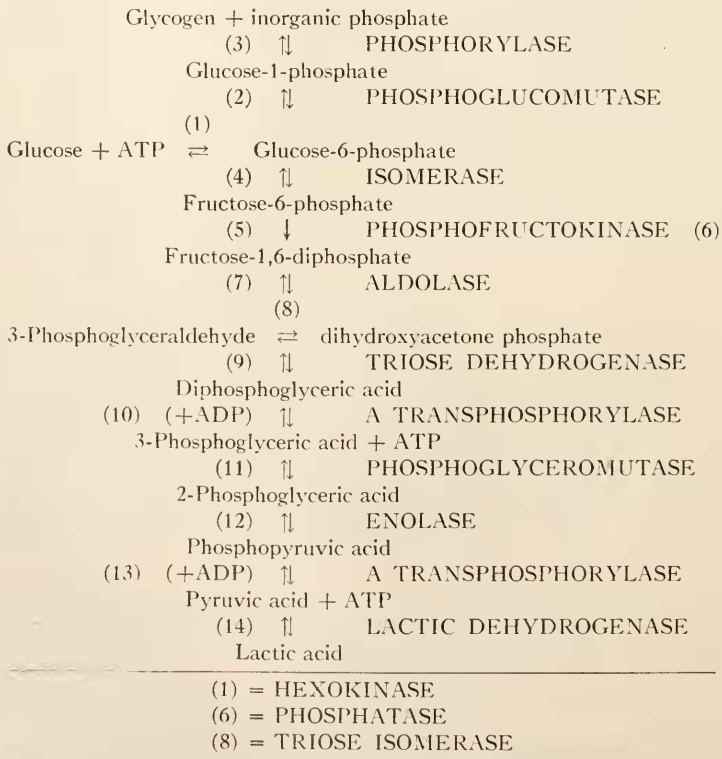

FIGURE 1. Steps in anaerobic breakdown of glucose or glycogen in animal tissues.

D. Metabolic Catalysts, Metabolic Intermediates; Phosphorus, Nucleic Acid and Calcium Metabolism; Echinochrome

1. Metabolic Catalysts. The search for metabolic catalysts in the sea urchin egg has been guided by previous observations on yeast and skeletal muscle. According to current concepts, carbohydrate is apparently broken down by muscle in two phases; in the first (anaerobic) phase, glucose or glycogen is broken down to pyruvate or to a 2 carbon fragment (Fig. 1). In the second (aerobic) phase, the carbons of the pyruvate or 2 carbon fragment are converted to carbon dioxide via the reactions of the tricarboxylic acid cycle (Fig. 2). The hydrogens removed during the aerobic phase are transferred, over diphosphopyridine nucleotide or triphosphopyridine nucleotide, flavin-adenine dinucleotide, and the cytochromes to react finally with oxygen and form water (Fig. 3); the hydrogens removed during the anaerobic cycle are used to reduce pyruvate to lactate. Amino acids employed as foodstuffs are first deaminated and then oxidized over the tricarboxylic acid cycle. Fatty acids to be oxidized are broken down to 2 carbon fragments which then also enter the tricarboxylic acid cycle (see Sumner and Somers, 1947; Krebs, 1943; Ball, 1944; and Lardy, 1949, for further discussion of biological oxidations).

During the sequence of events summarized in Figs. 1–3 energy is liberated by oxidation of the foodstuff. It has been postulated with the enzymes of kidney or liver that some 60 per cent of the energy derivable by complete oxidation of carbohydrate can apparently be stored up in the form of so-called "high energy" phosphate bonds (Lipmann, 1941; Hunter and Hixon, 1949). The terminal group of adenosine triphosphate is such a bond and this group can be transferred to synthesize other more stable phosphate compounds or to create active intermediates in the synthesis

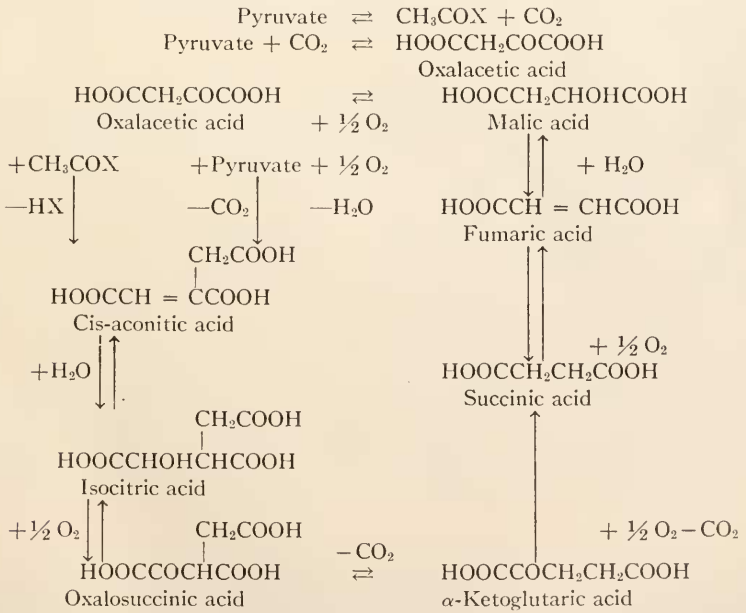

Figure 2. Some possible steps for pyruvate utilization via the tricarboxylic acid cycle.

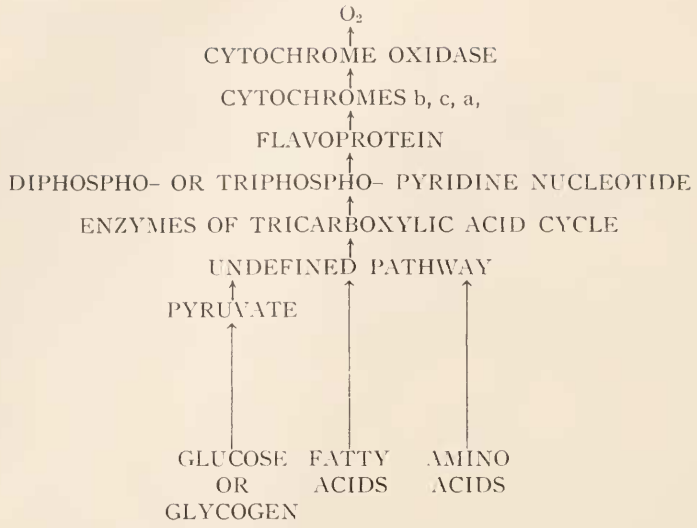

FIGURE 3. Schematic outline of steps by which hydrogens (or electrons) of foodstuffs react with oxygen in animal tissues to form water.

of many cellular constituents. From these facts it is postulated that the major portion of the energy used for synthesis and function must pass through such a mechanism. Most of these "high energy" phosphate bonds are created during the aerobic phase of substrate disposal mentioned above.

None of the enzymes for the anaerobic steps shown in Figure 1 has been studied in Arbacia eggs, nor have specific enzymes for deamination of amino acids or breakdown of fatty acids been looked for.

a. Cytochrome oxidase. Arbacia eggs contain an enzyme which is similar to the cytochrome oxidase of other animal tissues, in that it oxidizes reduced cytochrome c, the cytochrome c concentration for half maximal activity being 4×10^{-6} *M*. The enzyme is reversibly inhibited by carbon monoxide; it is also inhibited by low concentrations of other iron-binding reagents such as cyanide, azide, and sulfide; it is not inhibited by copper-binding reagents such as 8-hydroxyquinoline or diethyldithiocarbamate. This enzyme occurs in nearly equal amounts in unfertilized and fertilized Arbacia eggs, being present in both in a concentration sufficient to account for the respiration of the fertilized eggs even under the maximum respiratory stimulation yet observed (Krahl, Keltch, Neubeck, Clowes, 1941).

Measurements with the Cartesian diver upon centrifugally isolated fractions show the cytochrome oxidase of Arbacia eggs to be associated with the small particulate elements of the cytoplasmic matrix (Hutchens, Kopac, Krahl, 1942) rather than with the particles stainable with Janus green which are customarily referred to as mitochondria (Harvey, 1941).

b. Cytochromes and related compounds. No cytochrome c has been found in Arbacia eggs (Ball and Meyerhof, 1940; Krahl, Keltch, Neubeck, Clowes, 1941). The latter workers used a concentrating procedure which was shown to detect 2

micrograms of added cytochrome c per gram of eggs. This quantity would be equivalent to about 2×10^{-7} *M* in the egg cytoplasm; since the measurements referred to above indicate that the cytochrome oxidase of the egg is fully active only at concentrations above 10^{-5} *M*, and only 10 per cent active at 10^{-6} *M*, considerable doubt arises as to a possible role for cytochrome c in the Arbacia egg.

Ball and Meyerhof found, in the Arbacia egg, iron porphyrin compounds related to the active group of cytochrome c. Cytochromes a and b have not been demonstrable on the basis of spectroscopic observations.

Cytochrome c is plentiful in Arbacia sperm (Ball and Meyerhof, 1940).

c. Flavin-adenine dinucleotide. Flavin-adenine dinucleotide has been extracted from Arbacia eggs and identified by its ability to serve as the prosthetic group of d-amino acid oxidase and by the quantitative absorption spectrum of the lumiflavin obtained from it (Krahl, Keltch, Clowes, 1940). The average content is about 10 micrograms per gram wet eggs, no consistent change with fertilization and development being noted.

d. Diphosphopyridine nucleotide (DPN). DPN has been extracted from the eggs with warm dilute acid, and assayed in terms of its ability to serve as the active group of phosphoglyceraldehyde and glycerophosphate dehydrogenases (Jandorf and Krahl, 1942). The apparent content of DPN in Arbacia eggs at various times after fertilization is, as micrograms per gram wet eggs: unfertilized, 385; fertilized 30 minutes, 345; fertilized 10 hours, 242. With modified extraction procedures, to preserve the acid labile (presumably dihydro-) form of DPN, the total DPN remains constant during the first 10 hours of development at about 300–400 micrograms per gram of eggs.

e. Enzymes of the tricarboxylic acid cycle. The experimental evidence currently available for mammalian tissues indicates that a cycle of reactions (similar to that illustrated in Fig. 2) may provide the mechanism by which the two carbon or three carbon fragments arising from partial breakdown of carbohydrate, amino acids, or fatty acids are converted to carbon dioxide with liberation of energy in a form which cells can use for synthetic reactions or for function. The structure of the initial condensation product arising from pyruvate and oxalacetate is not settled; it may be cis-aconitate, citrate, or possibly some other substance. Pyruvate is oxidized during a sequence of reactions to give three CO_2 molecules per pyruvate. One CO_2 is liberated during formation of the initial six carbon product of condensation, one during the oxidation of oxalosuccinic acid, and one during oxidation of α-ketoglutaric acid to succinic acid. By use of the enzymes from kidney, it has been demonstrated that about 15–20 atoms of inorganic phosphorus are converted into a "high energy" form during oxidation of each pyruvate, most, if not all, during and subsequent to the oxidation of α-ketoglutarate (Ochoa, 1943; Lehninger and Smith, 1949; Hunter and Hixon, 1949).

Efforts to demonstrate the role of the tricarboxylic acid system in Arbacia eggs have so far been confined to determining, first, whether pyruvate can be oxidized by the eggs and, second, whether oxalacetate, α-ketoglutarate, or succinate can be oxidized and, if so, whether "high energy" phosphate is generated thereby.

Oxidation of pyruvate. In most cells both the condensation and the decarboxylation reactions of pyruvate apparently require diphosphothiamine, the biologically active form of vitamin B_1 (Barron and Lyman, 1940).

The diphosphothiamine content of the Arbacia egg was assayed on the basis of the ability of this substance to serve as the active group of yeast decarboxylase. Both Krahl, Jandorf, and Clowes (1942), and Goldinger and Barron (1946) found Arbacia eggs to contain 2–3 micrograms diphosphothiamine per gram wet eggs; this value was not significantly altered by fertilization.

Goldinger and Barron (1946), using a chemical method for pyruvate estimation, found about 70 micrograms of pyruvate per gram dry weight of unfertilized or fertilized Arbacia eggs. They determined the rate of disappearance of pre-formed pyruvate in intact unfertilized and fertilized Arbacia eggs; the rates of pyruvate disappearance at 20° C. were found to be: unfertilized, 66 γ, fertilized, 456 γ, per hour per gram dry weight; these values correspond to 0.2 and 1.3 μM per hour per gram wet weight. Krahl, Jandorf, and Clowes (1942), using a specific but relatively less accurate enzymatic method for pyruvate estimation, determined the rate of disappearance of pyruvate added to cytolysates of unfertilized and fertilized eggs; their values, in μM per gram wet eggs per hour at 20° C., were: unfertilized, 1.1; fertilized, 1.5. It will be noted that the rates for the eggs and for cytolysates are nearly the same after fertilization, but that breakdown of egg structure has raised the rate for cytolysates of unfertilized eggs closer to the fertilized level.

Neither Goldinger and Barron nor Krahl, Jandorf, and Clowes determined the fate of the pyruvate or whether its oxidative removal was coupled with generation of "high energy" phosphate bonds.

Relation of oxidation of oxalacetate, α-ketoglutarate, and succinate to phosphorylation. A cell-free particulate system has been prepared from unfertilized Arbacia eggs which can use substrates of the tricarboxylic acid cycle to effect phosphorylation (Keltch, Strittmatter, Walters, and Clowes, 1949, 1950). Although the effect of these substrates on oxygen consumption is small, owing to a large baseline respiration, phosphorylation is specifically made possible by their oxidation. The phosphorylation is increased from 130 γ per hour in absence of added substrate to 1010 γ with α-ketoglutarate, 910 γ with oxalacetate, and 530 γ with succinate, all values being for a quantity of homogenate obtained from 1 gram wet unfertilized eggs. The ratio: atoms P esterified/atoms oxygen consumed was found to be 1.1 with α-ketoglutarate as substrate.

To prepare a cell-free particulate system capable of oxidizing these substrates, it is necessary to minimize breakdown of the pigment granules during homogenization, as echinochrome apparently inactivates one or more of the catalysts required (Crane and Keltch, 1949). Echinochrome is a substituted naphthoquinone (see below) capable of oxidizing reduced sulfhydryl groups which are known to be essential for oxidation of succinate and other substrates (Hopkins and Morgan, 1938; Barron and Singer, 1943). Earlier unsuccessful attempts to demonstrate oxidation of α-ketoglutarate (Goldinger and Barron, 1946) or succinate (Ball and Meyerhof, 1940; Ballentine, 1940; Goldinger and Barron, 1946) may be due in part to inactivation of the enzymes in egg extracts by echinochrome, and in part to failure of these substances to penetrate into intact eggs.

Homogenates capable of effecting oxidative phosphorylation have been prepared from unfertilized *Strongylocentrotus purpuratus* eggs by Lindberg and Ernster

(1948). Pyocyanine and DPN were added as electron carriers and glucose was added as a supplemental substrate.

f. Oxidation of other substrates. Ballentine (1940b) found, in Arbacia egg cytolysates, no evidence for oxidation of the following substrates: glucose, lactate, α-glycerophosphate, succinate, or butyrate.

Homogenates of other echinoderm eggs have been observed to oxidize a number of substrates in the presence of pyocyanine or other dyes as hydrogen acceptors (see Runnström, 1949, for review). In particular, Lindberg (1943) found that homogenates of Echinocardium eggs could catalyze the reduction of methylene blue by malic, formic, or phosphogluconic acids or by propanediol phosphate. The latter substance was studied because Lindberg isolated a phosphate fraction from sea urchin eggs corresponding in properties to propanediol phosphate. Lindberg (1943) has suggested that part of the energy for development of sea urchin eggs may be derived from oxidation of glucose-6-phosphate over the triphosphopyridine nucleotide-flavoprotein pathway (Warburg and Christian, 1936; Dickens, 1938).

g. Hydrolytic enzymes.

(1) *Adenosine triphosphatase and ATP.* This enzyme, which hydrolyzes adenosine triphosphate (ATP), has been found in eggs of *Strongylocentrotus purpuratus* (Connors and Scheer, 1947) and in *Paracentrotus lividus* (see Runnström, 1949); the ATP-ase activity of the fertilized Strongylocentrotus egg is reported to be higher than that for unfertilized, the amount of inorganic P liberated per hour per mg. egg nitrogen being 10.3 and 4.1 mg., respectively.

A substance similar to, or identical with, adenosine triphosphate was extracted from Arbacia eggs by White and Chambers (1949).

(2) *Phosphatase.* The acid phosphatase activity of the unfertilized Arbacia egg is 3–5 times that of the alkaline phosphatase, and remains constant during the first 40 hours of development; the alkaline phosphatase content remains constant until just before gastrulation and then rises to 10 times that of the unfertilized egg (Mazia, Blumenthal, Benson, 1948). Alkaline phosphatase activity toward the artificial substrate, glycerophosphate, was also studied at various stages of Arbacia development by Krugelis (1947).

(3) *Ribonuclease and desoxyribonuclease.* Evidence for ribonuclease activity in Arbacia eggs has been recorded by Bernstein (1949); Lansing and Rosenthal (1949) found some localization of ribonucleic acid at the surface of Arbacia eggs.

Mazia, Blumenthal, and Benson (1948) studied the distribution of desoxyribonuclease in the Arbacia egg. They found that in the unfertilized egg this enzyme is not restricted to nuclear structures, and that most of the desoxyribonuclease is in the cytoplasm, though no measurable amount of desoxyribonucleic acid is found there. During the first 40 hours of development after fertilization, there is no increase in the total desoxyribonuclease content of the egg, but the fraction of the enzyme which is non-sedimentable at 20,000 g. declines as development proceeds, being about 10–20 per cent of the total activity at 40 hours.

(4) *Peptidases.* Holter and co-workers (1936, 1938) studied the peptidase activity toward alanylglycine of unfertilized Arbacia eggs and fractions prepared from them by centrifuging. Holter found that the peptidase is localized in the hyaline ground substance (matrix) of the egg cytoplasm and not bound to granular matter.

Doyle (1938) found the peptidase content of *Psammechinus miliaris* to be the same before and after fertilization, in agreement with the experiments of Holter (1936) upon Echinarachnius.

Lundblad (1949) demonstrated proteolytic activity toward gelatin in eggs and sperm of *Arbacia lixula* and sperm of *Paracentrotus lividus*.

h. Catalase. Catalase activity was found in eggs and egg fragments of Psammechinus by Doyle (1938). Fertilized eggs with intact membranes showed lower catalase activities than unfertilized eggs. Fertilized eggs with membranes destroyed showed the same catalase activity as unfertilized eggs. (For earlier observations on catalase in Arbacia eggs, see Harvey review, 1932.)

2. Metabolic Intermediates. During the first eight hours of development, the synthetic activities of the sea urchin egg consist chiefly in rearranging the nuclear and cytoplasmic components, with little or no increase in the total organic mass of the egg (Gray, 1927; E. B. Harvey, 1949). The question of what foodstuffs are available and used to provide energy for these synthetic activities according to the reactions illustrated in Figures 1–3 has been given only preliminary study in *Arbacia punctulata* eggs, and has received somewhat more attention in European sea urchins (see Örström and Lindberg, 1940; and Lindberg, 1946).

a. Total acid-hydrolyzable polysaccharide. This was reported to be equivalent to 50 mg. glucose per gram Arbacia egg protein by Perlzweig and Barron (1928), and 110 mg. glucose per gram egg protein by Hutchens, Keltch, Krahl, and Clowes (1942). The discrepancy was attributed by the latter authors, in part, to differences in the time of the season at which the eggs were collected and used, as they found that the carbohydrate values were higher from stored than from recently collected urchins. Confirming this suggestion, it was later found that the nitrogen content of the Arbacia egg tends to fall during the latter part of the season, especially if urchins are stored for several days or weeks in indoor aquaria (Crane, 1947). This nitrogen loss tends to elevate analytical values which are based on egg nitrogen or protein content.

Of the acid-hydrolyzable polysaccharide, only a negligible amount comes from the jelly (Hutchens, Keltch, Krahl, and Clowes, 1942).

No free reducing sugar was found by either group of investigators.

b. Glycogen. Of the 110 mg. total acid-hydrolyzable polysaccharide per gram egg protein found by Hutchens, Keltch, *et al.* (1942), 46 per cent was isolated as an alkali-stable, alcohol-precipitable polysaccharide. This substance corresponds to typical animal glycogen in optical rotation, yield of glucose upon hydrolysis, and reaction with iodine. This glycogen content is of the same order as that (50–80 mg. per gram egg protein) obtained for Arbacia by Blanchard (1935) and for the European sea urchins *Paracentrotus lividus* and *Echinus esculentus* (see Örström and Lindberg, 1940, for a summary of the total carbohydrate and glycogen contents of these eggs).

c. Other possible metabolic intermediates. Other metabolic intermediates indicated in Figures 1 and 2 have not been looked for in Arbacia eggs, but the phos-

phate fractions of European sea urchins have been studied by Zielinski (1939) and Lindberg (1943). In Echinocardium eggs, the latter reported that there are only three principal forms of phosphate; 53 per cent of the total being inorganic orthophosphate, 31 per cent adenosine triphosphate, and 16 per cent propanediolphosphate.

d. Fats. The total fat in 10^6 unfertilized eggs is 5.65 mg., of which 7.5 per cent is sterol and 38 per cent phospholipid. After fertilization, the total decreases up to 8 hours, increases to 20 hours, and then declines again up to 40 hours (Hayes, 1938).

Parpart (1941) found the total lipid to be 5.4 per cent of the whole egg, or 26.9 per cent of egg solids; 77 per cent of the lipid appeared to be bound to protein. Neither the total lipid nor lipid bound to protein was found to change during the first 5 hours of development.

Navez and E. B. Harvey (1935), Navez (1939), and Navez and DuBois (1940) have studied the unsaturated fatty acid fraction of Arbacia eggs, some component of which acts as a catalyst in oxidation of the Nadi reagent, thus simulating the enzyme indophenol oxidase.

e. Foodstuffs consumed and products formed during cleavage. Hutchens, Keltch, Krahl, and Clowes (1942) measured the glucose used, and the lactic acid, carbon dioxide, and ammonia formed by the fertilized Arbacia egg during the first few hours after fertilization. Their observations are as follows: (1) little or no lactic acid is formed; (2) the glucose used is, in 5 of 6 experiments, not adequate to account for the oxygen consumed; (3) the oxygen required for protein oxidation (calculated on basis of ammonia produced) is sometimes greater, sometimes less, than the oxygen consumption not accounted for by carbohydrate oxidation. For the periods in which the total oxygen consumption could not be accounted for by the sum of apparent carbohydrate and protein oxidation, the respiratory quotients are low, suggesting that fat may be oxidized.

Perlzweig and Barron (1928) had previously measured the lactic acid content before and immediately after fertilization.

Zielinski (1939) and Örström and Lindberg (1940) found a decrease in carbohydrate content of various sea urchins to occur upon fertilization, but they did not study the period 0.5–3 hours after fertilization, so that their results cannot be directly compared with those of Hutchens, Keltch, Krahl, and Clowes (1942).

3. Phosphorus and Nucleic Acid Metabolism in Arbacia Eggs. Determinations of the total phosphorus of the Arbacia egg and its partition are given in Table II.

Measurements of the rate of uptake of inorganic P^{32} from sea water by Arbacia were made by Abelson (1947, 1948). He found the total P^{32} uptake by fertilized eggs in a given time to be 40 times that of the unfertilized eggs; the amount taken up by fertilized eggs at 10° C. was only one-seventh that at 23° C. Of the total P^{32} taken up by fertilized eggs, 97 per cent appeared in the acid-soluble fraction. Of this acid-soluble fraction, 16 per cent was inorganic phosphate, 41 per cent adenosine triphosphate and diphosphate, 41 per cent barium-soluble, and 2 per cent unaccounted for. These measurements were made using carriers for inorganic

TABLE II

Analyses of the phosphorus fractions of Arbacia eggs, milligrams P per gram wet weight egg or embryo (modified from Villee, Lowens, Gordon, Leonard and Rich, 1949)

Investigator	Stage	Acid soluble P	Phospho-lipid P	Acid insoluble P	DNA P	RNA P	Phospho-protein P	Total P
Crane (1947)	unfert. eggs	1.3	1.3	0.86	0.03			3.2 [1]
Schmidt, Hecht, and Thannhauser (1948)	unfert. eggs	—	1.5	1.0	0.04	0.86	0.11	4.1
Villee *et al.* (1949)	3 hrs. embryos	1.4	—	0.86	0.05	0.74	0.06	—

[1] Analyses for individual fractions were carried out on separate batches of eggs, and the original values were based on egg nitrogen content. Later, Crane found that the nitrogen content of various batches of eggs decreased with increasing periods of storage of the urchins. For this reason, the total P is not equal to the sum of the individual fractions.

phosphate and ATP, and were recorded with some reserve because of interference of echinochrome with the phosphate fractionation.

The rates at which inorganic P^{32} enters the various organic fractions of the fertilized Arbacia egg were determined by Villee, Lowens, Gordon, Leonard, and Rich (1949). After 6 hours of development, the relative quantities of P^{32} in the various fractions were (expressed as counts per minute per mg. $P \times 10^3$): acid soluble—P, 20; RNA—P, 61; acid insoluble—P, 75; DNA—P, 109; phosphoprotein—P, 135. On the basis of the fact that P^{32} enters the DNA more rapidly than the RNA fraction, they conclude that the DNA of the fertilized egg is not formed from the RNA of the unfertilized egg, as had been suggested for eggs of *Paracentrotus lividus* by Brachet (1947).

The P^{32} uptake by other sea urchin eggs has also been measured. Lindberg (1949) has determined the rate at which P^{32} is incorporated into the acid-soluble, acid-labile fraction (presumed by Lindberg to be adenosine triphosphate) of Psammechinus eggs. In the unfertilized eggs, the initial rate of P^{32} uptake into this fraction is low, and a steady state is reached within 5 minutes after exposure to the P^{32}. In the fertilized egg, the rate of P^{32} incorporation into this fraction is much higher, and is sustained for at least 60 minutes after fertilization.

Measurements of P^{32} uptake have been made upon *Strongylocentrotus* (*purpuratus* and *franciscanus*) by Brooks and Chambers (1948), Chambers and White (1949), and upon *Lytechinus pictus* by Chambers, Whiteley, Chambers, and Brooks (1948).

Schmidt, Hecht, and Thannhauser (1948) reported that the unfertilized *Arbacia punctulata* egg contains 20×10^{-5} micrograms ribonucleic acid—P(RNA–P) and 1×10^{-5} micrograms desoxynucleic acid—P(DNA–P) per egg, i.e. 0.86 and 0.04 mg. per wet gram of eggs, respectively. During the first 24 hours of development, the amount of ribonucleic acid per embryo remains virtually unchanged, but the desoxynucleic acid steadily increases until, at the end of this period, the desoxyribosenucleic acid content per embryo is 10–15 times that of the unfertilized egg.

Blanchard (1935) had previously isolated 1.08 grams of a relatively pure sample of desoxynucleic acid from 4820 grams of wet unfertilized Arbacia eggs; he also isolated a small amount of ribosenucleic acid.

4. Calcium Metabolism. Heilbrunn, Mazia, and Steinbach (1934) and Mazia (1937) have found that the proportion of bound calcium ion in Arbacia eggs decreases by about 15 per cent on fertilization, the total calcium remaining constant. Mazia calculated that the free calcium of the unfertilized eggs is about 0.0005 molar, increasing by 0.001 molar upon fertilization. The relation of calcium ion to activation processes has been discussed by Heilbrunn (1943).

Changes in free calcium in other echinoderm eggs have been measured by Örström and Örström (1942) and Monroy-Oddo (1946). Hultin (1949) found that calcium ion increased both oxygen uptake and anaerobic acid formation in homogenates of Psammechinus eggs.

5. Echinochrome. The precise function of echinochrome in metabolism of the Arbacia egg has yet to be defined. The observations of Keltch and co-workers (1949, 1950), recorded above, suggest that echinochrome, when it leaves the pigment granules and gains access to the egg cytoplasm, may inactivate enzymes which contain sulfhydryl groups.

Crystalline echinochrome was isolated from the eggs and tests of *Arbacia punctulata* by Ball (1936). The pigment is capable of reversible oxidation-reduction and has a potential, E_0' at pH 7 and 30° C., of -0.221 V. and an E_0 of $+0.1995$ V. (Cannan, 1927). The structure of echinochrome from *Arbacia punctulata* eggs has not been determined, but that from *Arbacia lixula* eggs, which is presumed to be similar to that from *Arbacia punctulata*, has been shown to be (in the reduced form) 1,3,4,5,6,7,8-heptahydroxy-2-ethylnaphthalene (Kuhn and Wallenfels, 1939).

The spectrophotometric properties for echinochrome from the eggs are the same as for that from the amoebocyte and test of *Arbacia punctulata* (Ball and Cooper, 1949). Echinochrome has an acid group of pK′ 6.38 at 26° C. The acid form has 3 absorption peaks at 255, 335, 475 mμ, with molecular absorption coefficients of 1.93×10^4, 0.87×10^4, 0.65×10^4, respectively. The three peaks for the ionized form are centered at 275, 400, and 475 mμ with molecular absorption coefficients of 2.11×10^4, 0.88×10^4, and 1.12×10^4, respectively. The eggs contain on the average 0.58 gram echinochrome ($C_{12}H_{10}O_7$) per 100 cc. of eggs packed by centrifuging.

E. Penetration of Chemical Agents into Arbacia Eggs

The effects of many weak acids and bases upon Arbacia eggs are strongly dependent upon the pH of the sea water medium in which the eggs are suspended.

Detailed analyses of the relation of pH to intensity of physiological effect in Arbacia eggs have been made for carbon dioxide (Smith and Clowes, 1924; Haywood and Root, 1930, 1932); for substituted phenols (Krahl and Clowes, 1938; Hutchens, Krahl, and Clowes, 1939); for barbiturates (Krahl, 1940; Clowes, Keltch, and Krahl, 1940); and for local anesthetics (Krahl, Keltch, and Clowes, 1940).

The total concentration of each phenol, barbiturate, or local anesthetic required to produce 50 per cent inhibition of cleavage was measured at each of several extracellular pH values, and the concentration of undissociated molecules and ionized molecules was calculated in the conventional way from the equation:

$$pH = pK'_a + \log \frac{\text{anion concentration}}{\text{undissociated molecule concentration}}$$

for the weak acids and the analogous equation:

$$pH = pK'_{water} - pK'_b - \log \frac{\text{cation concentration}}{\text{undissociated molecule concentration}}$$

for bases, where pK'_a, pK'_b, pK'_w are the negative logarithms of the dissociation constants of the acid, the base, or water, respectively. It was found, with any one agent, that the concentration of *undissociated molecules* required to produce 50 per cent inhibitition of the rate of cleavage is the same at various extracellular pH values, whereas neither the total concentration nor the ion concentration remains constant as the pH is varied. An example is shown in Table III. These observations were

TABLE III

Concentration of undissociated substituted phenol molecules required to produce 50 per cent reduction of the rate of cell division of fertilized eggs of Arbacia punctulata at pH 6.6 and pH 8.1 in glycyl glycine buffer (Krahl and Clowes, 1938a)

Temperature 20° C.

Substituted phenol	pK′	Total concentration		Extracellular molecule concentration	
		pH 6.6	pH 8.1	pH 6.6	pH 8.1
		moles per l.	moles per l.	moles per l.	moles per l.
2,4-dinitrophenol	4.1	3.4×10^{-6}	6.3×10^{-6}	1.1×10^{-8}	0.6×10^{-8}
4,6-dinitro-o-cresol	4.4	8.0×10^{-7}	1.5×10^{-5}	5.1×10^{-9}	3.8×10^{-9}
2,4,5-trichlorophenol	6.9	1.7×10^{-6}	1.5×10^{-5}	1.1×10^{-6}	1.1×10^{-6}
2,4-dichlorophenol	7.7	3.2×10^{-5}	1.1×10^{-4}	3.0×10^{-5}	3.1×10^{-5}
m-nitrophenol	8.3	1.7×10^{-4}	3.2×10^{-4}	1.7×10^{-4}	2.0×10^{-4}

interpreted to indicate that these weak acids and bases penetrate Arbacia eggs principally, and in most cases entirely, in the form of undissociated molecules. In the course of these same studies, an attempt was made to identify the form in which these agents act within the cell.

Tyler and Horowitz (1937) found that the concentration of the anion of each of 8 substituted phenols required to produce 90 per cent inhibition of cleavage of eggs of *Strongylocentrotus purpuratus* is the same, from which they concluded that the active agent in blocking cleavage was the anion. In contrast, Krahl and Clowes (1938), using a more complete series of 30 substituted phenols, upon Arbacia eggs, found that the calculated anion concentrations required to produce 50 per cent inhibition of cleavage vary from 0.1×10^{-6} *M* for 2,4-dinitro-o-cyclohexylphenol up to 192×10^{-6} *M* for 2,4-dinitro-o-isopropylphenol. The measured values from which these calculations were made are given in Table IV. Thus, the attrac-

tive hypothesis of Tyler and Horowitz is not supported by more extensive data upon Arbacia. Krahl and Clowes (1938) and Hutchens, Krahl, and Clowes (1939), after studying effects of these agents at varying intracellular and extracellular pH values, drew the tentative conclusion that the undissociated molecule was the form active in inhibiting cleavage, while the anion might be the active agent in stimulating oxygen consumption (see below).

Though not studied in detail, the variation in effects of other weak acids with pH is qualitatively like that of the agents mentioned above. The physiological effects upon Arbacia eggs of hydrogen cyanide (pK′ 9.2), hydrazoic acid (pK′ 4.7), hydrogen sulfide (pK′ 7.0), and iodoacetic acid (pK′ 3.1) are more marked in acid

TABLE IV

Effects of substituted phenols on oxygen consumption and cleavage of fertilized eggs of Arbacia punctulata at pH 7.5 and 20° C.

(From Clowes and Krahl, 1936; Krahl and Clowes, 1936, 1938a)

Substituted phenol	pK′ at 25° C.	Oxygen consumption, maximum rate, as per cent of normal [1]	Cleavage, total conc. to give 50 per cent inhibition
			moles per l. × 10^6
1. 2,4-dinitro-o-cyclohexylphenol	4.2	—	0.6
2. 2,4-dinitro-ar-tetrahydro-α-naphthol	4.5	320	1
3. 4,6-dinitro-2-(1,1-dimethylhexyl)-phenol	—	260	1
4. 2,4-dinitrothymol	4.1	no stimulation	1
5. 4-nitrocarvacrol	4.2	—	2
6. 2,4,6-triiodophenol	6.6	180	3
7. 4,6-dinitro-o-cresol	4.4	300	4
8. 2,4-dinitro-α-naphthol	3.6	230	6
9. 2,4,5-trichlorophenol	6.9	250	10
10. 2,4-dinitro-o-phenylphenol	3.9	—	10
11. 2,4,6-tribromophenol	6.0	230	16
12. 2,4-dinitrophenol	4.1	290	23
13. 4,6-dichloro-o-cresol	7.7	190	25
14. 2,6-dibromo-4-nitrophenol	3.7	240	25
15. 4,6-dinitrocarvacrol	4.5	360	46
16. 2,6-dichloro-4-nitrophenol	3.7	230	55
17. 2,6-dinitrophenol	3.7	270	55
18. 4,6-dibromo-o-cresol	7.6	230	98
19. 2,4,6-trichlorophenol	6.2	220	98
20. 2,4-dichlorophenol	7.7	230	100
21. p-nitrophenol	7.2	325	100
22. 2-hydroxy-5-chlorobenzaldehyde	7.4	—	125
23. 2,4-dibromophenol	7.8	270	138
24. 2-nitro-4-chlorophenol	3.5	250	184
25. 2-hydroxy-5-bromobenzaldehyde	7.4	—	250
26. m-nitrophenol	8.3	230	314
27. 2,6-dinitro-4-chlorophenol	3.5	210	400
28. 2,6-dibromophenol	6.6	120	900
29. 2,4-dinitro-o-isopropylphenol	3.0	—	1024
30. 2,4,6-trinitrophenol	0.7	no stimulation	6900
31. o-nitrophenol	7.2	no stimulation	22,600

[1] The concentrations required to produce maximal stimulation of oxygen consumption are about 0.5-1 times those required to produce 50 per cent inhibition of cleavage.

than in neutral or alkaline solutions (Clowes and Krahl, 1940). The sodium or potassium salts of such weak acids dissolve to give alkaline solutions which must be neutralized and buffered in the physiological range.

These principles must be kept in mind in comparing measurements of various investigators who have, in many cases, taken insufficient account of the effect of pH upon penetration of the agent under study.

F. Effects of Chemical Agents upon Oxygen Consumption, Cleavage, and Metabolic Enzymes

Effects of chemical agents upon Arbacia eggs have been studied from a number of points of view. The two principal objectives, with examples of agents used to approach them, are: First, elucidation of the processes by which energy is provided for development of the egg—low oxygen tension, cyanide, carbon monoxide, azide, sulfide, inhibitors of copper catalysts, iodoacetate, substituted phenols, urethane, naphthoquinones, and dyes; second, analysis of the biochemical mechanism of action of physiologically active agents, including mechanism by which agents penetrate cell surfaces—substituted phenols, barbiturates, local anesthetics, xanthines, penicillins, sulfonamides, colchicine, podophyllotoxin, and a number of other agents.

1. Low Oxygen Tension. The problem of requirement of oxygen for cleavage of sea urchin eggs was first posed specifically by J. Loeb (1895); he found that Arbacia eggs could not undergo sustained cleavage when oxygen tension is reduced by replacement of air with hydrogen. More detailed study showed that cleavage is completely suppressed at a level of 0.4 per cent oxygen—99.6 per cent nitrogen, where the oxygen consumption is 20–30 per cent of normal (Amberson, 1928; Clowes and Krahl, 1940).

Cytological observations by E. B. Harvey (1930) and Fry (1937) indicate that detailed metabolic activities are blocked either immediately or within a few minutes after the eggs are brought under anaerobic conditions. This indicates that the Arbacia egg has neither a reserve energy source nor a mechanism for energy liberation upon which it can support cleavage when oxygen is lacking.

2. Cyanide, Carbon Monoxide, Azide, Sulfide, Substances forming Complexes with Copper. The effects of each of these agents have been tested upon egg respiration and cleavage, and upon cytochrome oxidase from the eggs.

Cyanide—In the unfertilized egg, about 50 per cent of the oxygen consumption is cyanide sensitive (Clowes and Krahl, 1934; Robbie, 1946).

Effects of cyanide upon oxygen consumption and cleavage in fertilized Arbacia eggs have been measured by a number of investigators (Table V). For other data which are not in a form adaptable to tabulation see Brooks (1933, 1941), Brinley (1930), Blumenthal (1930), Barron and Hamburger (1932), Shapiro (1940), Krahl and Clowes (1940).

Loeb (1906) observed that fertilized *Strongylocentrotus purpuratus* eggs would not divide in solutions of potassium cyanide. Apparently the first simultaneous measurements on both respiration and cleavage in presence of cyanide were made by Warburg (1910) upon eggs of *Strongylocentrotus lividus;* cleavage was blocked at a concentration of 0.0001 *M* cyanide, in which oxygen consumption was reduced

to about 20 per cent of normal. These observations by Warburg were the first in a long series which led eventually to his characterization of the *Atmungsferment* as a metalloprotein and to the isolation in pure form of the yellow enzymes (flavoproteins) and of triphosphopyridine nucleotide.

Cleavage of the fertilized Arbacia egg is blocked at 20° C. when oxygen consumption is reduced by cyanide to about 35 per cent of normal (Clowes and Krahl, 1940; Robbie, 1946), but the concentration required to produce this effect cannot be precisely stated, owing to the dependence of this concentration upon the conditions of measurement. Potassium cyanide, at pH values in the physiological range,

TABLE V

Effect of cyanide upon oxygen consumption and cleavage of sea urchin eggs

Investigator	Unfert. (U) or fert. (F)	Temp. °C.	pH of medium	Oxygen consumption			Cleavage, concentration required for 100 per cent inhibition
				Inhibition at cyanide conc. required for 100 per cent inhib. of cleavage	Degree of maximal inhibition	Conc. required for maximal inhibition	
				per cent of control	per cent of control	moles per l.	moles per l.
Warburg (1910)[1]	F	—	—	80	80	1×10^{-4}	1×10^{-4}
Loeb and Wasteneys (1911)[2]	F	20	—	75	—	—	4×10^{-5}
Loeb and Wasteneys (1913)[2]	F	14	—	67	—	—	3×10^{-5}
Barron and Hamburger (1932)[3]	F	24	8.2	—	100	1×10^{-3}	—
Clowes and Krahl (1934b)[3]	U	21	8.2	—	50	1.3×10^{-3}	—
Korr (1937)[3,4]	F	15	—	—	39	2.5×10^{-3}	—
		20	—	—	49	2.5×10^{-3}	—
		25	—	—	57	2.5×10^{-3}	—
Lindahl (1940)[5]	U	19–22	—	—	43	1×10^{-3}	—
	F	19–22	—	—	88	1×10^{-3}	—
Clowes and Krahl (1940)[3]	F	20	7.9	65	75	1×10^{-3}	1.6×10^{-4}
		24	7.9	—	84	1×10^{-3}	—
Robbie (1946)[3]	U	20	8.0	—	57	1×10^{-4}	—
	F	20	8.0	65	82	1×10^{-4}	1.5×10^{-5}

[1] *Strongylocentrotus lividus.*

[2] *Strongylocentrotus purpuratus.*

[3] *Arbacia punctulata.*

[4] Values are given for temperature from 11–26°. Only one cyanide concentration was used (2.5×10^{-3}M); this was stated to be higher than that required for maximum inhibition.

[5] *Paracentrotus.* Values are also given for oxygen tensions other than that in air.

is in equilibrium with HCN, which is volatile and distills into the alkali used to absorb CO_2 in respiratory measurements by the Warburg method. Robbie (1946) has given detailed procedures for minimizing this difficulty. The opposite situation obtains in the older measurements by the Winkler method where respiratory CO_2 was allowed to accumulate; here the pH tends to fall and make the required concentration of cyanide lower than it would be at pH 8. All methods agree in indicating that the critical concentration of cyanide to block cleavage of Arbacia eggs is about 10^{-5}–10^{-4} *M* in unbuffered sea water.

As the cyanide concentration is increased, the oxygen consumption passes through a minimum; at the maximally inhibiting cyanide concentration, the oxygen consumption of the fertilized eggs is reduced by some 75–80 per cent (Warburg, 1910; Clowes and Krahl, 1940; Robbie, 1946). The fact that Korr (1937) observed a smaller degree of inhibition (49 per cent at 20° C.) may be due to his use of a concentration of cyanide above that which gives maximum inhibition. Lindahl (1940) found the inhibition by 0.001 *M* cyanide to be greater at low oxygen tensions than at 100 per cent oxygen, and Robbie (1946) likewise found a greater inhibition by 10^{-4} *M* cyanide as oxygen tension was lowered.

Cytochrome oxidase activity of Arbacia eggs is strongly inhibited by low concentrations of sodium cyanide, 50 per cent at about 3×10^{-6} *M*, and 100 per cent at about 2×10^{-4} *M* under the conditions of these experiments (pH 6.9). The degree of inhibition by a given concentration of cyanide is not greatly altered by variation in concentration of cytochrome c over the range from 0.34×10^{-5} *M* to 1.36×10^{-5} *M* nor by use of p-phenylene diamine instead of hydroquinone as substrate (Krahl, Keltch, Neubeck, Clowes, 1941).

Carbon monoxide—Carbon monoxide has the specific property of entering into catalytically inactive light-reversible combination with the ferrous iron of certain iron-porphyrin compounds (Warburg, 1926; Keilin, 1930). Its effects on oxygen uptake by isolated tissues are considered at present to be entirely attributable to this mechanism; an inhibition by carbon monoxide is thus more strongly indicative of dependence of respiration upon iron-porphyrin compounds than inhibition by cyanide, azide, or sulfide, which can combine readily with other metal-containing pigments than ferric porphyrin compounds, and which can also act as reducing agents.

With $CO-O_2$ mixtures in the dark, cell division is approximately 50 per cent inhibited at 2 per cent oxygen—98 per cent CO; completely and reversibly inhibited at 0.7 per cent oxygen − 99.3 per cent CO, at which point oxygen consumption is about 30 per cent of that in the control (Clowes and Krahl, 1940). The complete cell division block occurs at the same level of oxygen consumption as when simple oxygen lack is the division inhibiting agency; hence carbon monoxide (in contrast to azide; see below) appears to have no effect on cleavage other than that arising from its effect on oxygen consumption.

Both the partial inhibition of oxygen consumption and the partial suppression of cleavage by mixtures of 1.6 per cent O_2—98.4 per cent CO, or 2.1 per cent O_2—97.9 per cent CO, are reversed by light (Clowes and Krahl, 1940).

Carbon monoxide produces a light-reversible inhibition of the cytochrome oxidase from Arbacia eggs (Krahl, Keltch, Neubeck, Clowes, 1941).

The inhibition constant, $K = \frac{n}{1-n} \cdot \frac{pCO}{pO_2}$, where n is the per cent inhibition in the dark and pCO and pO_2 are the respective carbon monoxide and oxygen partial pressures, was calculated. For the cell-free cytochrome oxidase system from Arbacia eggs the value is about 3.1–3.2, at either 0.34×10^{-5} *M* or 1.36×10^{-5} *M* cytochrome c and 0.02 *M* hydroquinone as oxidizable substrate; it is 4.5 at 1.36×10^{-5} *M* cytochrome c and 0.001 *M* hydroquinone. For the respiration of the intact fertilized Arbacia egg the value of this inhibition constant is about 60, as compared to 5 for yeast saturated with glucose as substrate (Warburg, 1927) and 21 for fertilized *Paracentrotus lividus* eggs (Runnström, 1930).

The light-reversible carbon monoxide inhibition of respiration and cleavage is perhaps the best, if not the sole, evidence for participation of an iron-porphyrin catalyst related to cytochrome oxidase in the sequence of oxidative reactions which yield energy to support cleavage. But the precise nature of this catalyst must be settled by further investigation, there being two anomalies to be explained: first, the very low concentration in the egg of cytochrome c, the usual substrate for the cytochrome oxidase system; and second, the greater apparent affinity of CO for cell-free cytochrome oxidase than for the CO sensitive enzyme of the intact egg. The CO inhibition constant may provide a valuable criterion for the identification of the substance which is actually oxidized by the cytochrome oxidase in the egg.

No inhibition of oxygen consumption of unfertilized Paracentrotus or Arbacia eggs by carbon monoxide can be demonstrated (Runnström, 1930; Brooks, 1941). Brooks (1941) reported that unfertilized Arbacia eggs consume carbon monoxide at the same rate, and fertilized eggs at one-third the rate, that they consume oxygen. Any such carbon monoxide consumption must be taken into account in a quantitative reexamination of the carbon monoxide inhibition constants, but it should be noted that correction for CO consumption would make the inhibition constant for the fertilized eggs even higher than that observed.

Azide—Only 50 per cent of the respiration of fertilized Arbacia eggs is insensitive to azide; moreover, 50 per cent inhibition of cleavage occurred at an azide concentration (3×10^{-3} *M* at pH 7.9) inhibiting respiration by only about 10 per cent; complete and reversible inhibition of cell division occurred at an azide concentration (5×10^{-3} *M*) inhibiting respiration by 50 per cent (Clowes and Krahl, 1940). When considered in conjunction with the effects of oxygen lack, cyanide, and carbon monoxide mentioned above, these measurements indicate that azide interferes with cleavage by a mechanism other than by simple reduction of oxygen consumption. A possible clue to the nature of this action is found in the observation that azide can block "high energy" phosphate transfer in a cell-free system (Loomis and Lipmann, 1949) and in yeast (Reiner and Spiegelman, 1947). Cytochrome oxidase of Arbacia is 50 per cent inhibited by 6.4×10^{-5} *M* azide at pH 6.8 and 20° C. (Krahl, Keltch, Neubeck, Clowes, 1941).

Sulfide—The effect of sodium sulfide on respiration and cell division of fertilized Arbacia eggs was determined in flasks of the Dixon and Keilin type, in which alkali is not present during the period of oxygen uptake, and distillation of volatile acids such as H_2S from the medium during the period of measurement does not occur. Cleavage is 50 per cent inhibited at a sulfide concentration (10^{-4} *M*) inhibiting respiration by only 10 per cent. Complete but not reversible inhibition of cleavage

occurs at a sulfide concentration ($2 \times 10^{-4} M$) inhibiting respiration by about 50 per cent. Thus, the hydrogen sulfide-sulfide system, like the hydrazoic acid-azide system, appears to have some effect on cleavage which is not attributable merely to its effect on oxygen uptake (Krahl, Keltch, Neubeck, Clowes, 1941).

Cytochrome oxidase of Arbacia eggs is also inhibited by sodium sulfide, complete suppression of activity being produced at approximately $1 \times 10^{-3} M$ sodium sulfide at pH 6.9. But at higher concentrations, sulfide is rapidly oxidized by the egg cytochrome oxidase preparation, so that the precise inhibition-concentration relationships are difficult to establish.

Substances forming copper complexes—In view of the suggestion of Keilin and Hartree (1938) that cytochrome oxidase may be a copper compound, effects of a number of inhibitors of copper catalysts on egg respiration and cleavage and on Arbacia cytochrome oxidase were explored. Sodium diethyldithiocarbamate and 8-hydroxyquinoline did not inhibit either oxygen consumption of fertilized eggs or the cytochrome oxidase therefrom up to concentrations of $5 \times 10^{-3} M$. Potassium dithiooxalate ($10^{-3} M$), diphenylthiocarbazone ($10^{-4} M$), isonitrosoacetophenone ($2 \times 10^{-3} M$), or 8-hydroxyquinoline ($5 \times 10^{-5} M$) did not inhibit cleavage of the fertilized eggs. Diethyldithiocarbamate blocked cleavage irreversibly at a concentration of $3 \times 10^{-5} M$ (Table VIII); at higher concentrations, cleavage proceeded through the 16 cell stage, but the eggs were irreversibly injured, as they did not survive to swimming forms when returned to sea water at this point. This anomalous effect of the dithiocarbamates may be exerted by a mechanism other than combination with copper (Clowes and Krahl, 1940; Krahl, Keltch, Neubeck, Clowes, 1941).

3. Substituted Phenols and Related Substances. The effects of these substances upon oxygen consumption and cleavage of Arbacia eggs have been intensively studied by Krahl, Clowes, Keltch, and their collaborators (1936, 1938, 1940, 1949). As the effects of these substances upon enzyme systems have proved to be relatively specific (see below), their effects upon Arbacia eggs may be given in some detail.

a. Effects on eggs. The typical effect is illustrated by the behavior of 4,6-dinitro-o-cresol. As the concentration is increased, oxygen consumption is increased to a maximum at a concentration of about $4 \times 10^{-6} M$. Above this concentration, oxygen consumption drops off, and a reversible complete block to cleavage sets in at about $2 \times 10^{-5} M$, at which point oxygen consumption is still above normal (Clowes and Krahl, 1934a, 1936). Ellis (1933) had previously noted that first cleavage of fertilized eggs of *Urechis caupo* was delayed by 0.001 per cent 2,4-dinitrophenol.

These effects are not dependent on reversible oxidation-reduction of the nitro groups, as halogenated phenols act like nitrophenols with respect to both respiration and cleavage of fertilized Arbacia eggs (Krahl and Clowes, 1936).

The concentrations required to stimulate respiration and to block cleavage of fertilized Arbacia eggs are summarized for 30 substituted phenols in Table IV. From these data it can be seen that these agents vary substantially in their ability to inhibit cleavage, whether total concentration, undissociated molecule concentration, or anion concentration be used as the index of effectiveness (Krahl and

Clowes, 1938). In general, the concentration required to produce 50 per cent inhibition of cleavage is equal to, or slightly larger than, that which produces optimum respiratory stimulation.

The active substituted phenols also stimulate oxygen consumption of unfertilized Arbacia eggs (Clowes and Krahl, 1934b, 1936) and of artificially fertilized eggs (Keltch and Clowes, 1947). In the latter case the optimum level of oxygen uptake attained is approximately the same as that produced by the same agent upon sperm-fertilized eggs; the cleavage of the artificially fertilized eggs is blocked at approximately the same concentrations of 4,6-dinitrocresol or 2,4,5-trichlorophenol as those required for sperm-fertilized eggs (Keltch, Walters and Clowes, 1947).

Stimulation of oxygen consumption of unfertilized eggs by substituted phenols has not resulted in artificial parthenogenesis, as measured by appearance of a fertilization membrane.

Inhibition by nitrophenols of sperm fertilization requires concentrations much higher than those which block cleavage (Clowes, Krahl and Keltch, 1937).

Observations upon related compounds which do not exhibit the typical substituted phenol effect upon respiration and cleavage of Arbacia are summarized by Clowes and Krahl (1936).

For combined actions of substituted phenols, carbon monoxide, and other agents, see Krahl and Clowes (1940).

Cytological studies by Fry (1935) showed that eggs first exposed to an active substituted phenol at the metaphase, anaphase, telophase, or resting stages apparently tended to cease development in the subsequent prophase. Fertilized eggs suitably exposed to cyanide or x-radiation also tended to accumulate in the subsequent prophase. At first sight, this suggested that all these agents had some especially intense action at prophase. To attempt to settle this point, Fry and Krahl (1936) constructed a mitotic time schedule which would indicate the proportion of eggs in all mitotic stages at various times after fertilization. This enabled estimates of the average *normal* time spent by any given egg in a given mitotic stage to be made, and showed that this time was much longer for prophase than for any other phase, as was well known by qualitative observations upon the living egg. When the results with substituted phenols were quantitatively compared with this mitotic time schedule, it was found that the time spent by the inhibited eggs in prophase was perhaps *slightly* greater (relative to normal) than for other phases, but that the accumulation of prophase figures was principally due to the long duration of prophase and the resultant greater chance of trapping eggs at this stage. These considerations should be kept in mind in postulating a specific effect of any agent upon a specific stage in mitosis.

A more detailed general discussion of the substituted phenols has been given by Clowes (1948).

b. Effects on enzymes. The mechanism of the two phases of the substituted phenol action on Arbacia eggs, namely, the stimulation of oxygen consumption on the one hand and the inhibition of cleavage on the other, has received intensive study.

The analysis of the first phase of the effect was long hampered by the fact that the oxidative stimulation was attainable only with intact cells. No augmentation of activity of any soluble dehydrogenase enzyme, flavoprotein, cytochrome, or

cytochrome oxidase system by the substituted phenols has yet been found. The possibility that the substituted phenol anion might act like arsenate (Warburg and Christian, 1939) in substituting for phosphate in the triose phosphate dehydrogenase system was tested by Krahl (1947) with negative results. The current hypothesis is that the substituted phenols increase oxygen consumption by changing the relationship of one or more oxidative enzymes to the cell structure, in a way which gives a higher than normal overall rate of hydrogen (electron) transfer; the precise nature of this effect is not yet known (Krahl and Clowes, 1938b; Peiss and Field, 1948; Tepley, 1949). A foundation for further analysis of this oxidative stimulation has been laid by the observation that some stimulation of oxygen consumption by these agents can be obtained in cell-free granule systems from kidney (Loomis and Lipmann, 1948) and from Arbacia eggs (Crane and Keltch, 1949; Clowes, Keltch, Strittmatter, and Walters, 1949, 1950).

Efforts to account for the second phase of the substituted phenol effect, namely, the progressive inhibition of both oxygen consumption and cleavage in concentrations above the respiratory optimum, have revealed that these agents inhibit the following enzymatic activities: d-amino acid oxidase (Krahl, Keltch and Clowes, 1940), *Zwischen ferment* and cytochrome reductase (Haas, Harrer, and Hogness, 1942), phosphate accumulation and transfer by yeast cells (Hotchkiss, 1944; Spiegelman and Kamen, 1946). The concentrations required for inhibition in these systems were 10–100 times those required to inhibit cleavage of Arbacia eggs. Neither the cytochrome oxidase of Arbacia eggs (Krahl, Keltch, Neubeck, and Clowes, 1941) nor any water-soluble dehydrogenase is inhibited by physiologically active concentrations.

Observations which may be more pertinent to the supression of cleavage have just been reported by Clowes, Keltch, Strittmatter, and Walters (1949, 1950). They found that oxidative phosphorylation by a cell-free system from Arbacia eggs (see above) is inhibited by substituted phenols, in confirmation of the findings of Loomis and Lipmann (1948) upon kidney granules. The effects of the individual substituted phenols upon this cell-free phosphorylating system from the eggs are parallel to those of the same agents upon cleavage of the intact eggs in the following specific respects: (1) For each agent the block to both cleavage and phosphorylation is produced by concentrations just larger than that required for optimum oxygen consumption; (2) dinitrocarvacrol produces an increase in oxygen consumption in both the intact eggs and the particulate system, but blocks both cleavage and phosphorylation; on the other hand, its isomer, dinitrothymol, inhibits oxygen consumption in both systems and blocks both cleavage and phosphorylation; (3) p-nitrophenol stimulates oxygen consumption in both systems and blocks both cleavage and phosphorylation; its isomer, o-nitrophenol, is inactive toward oxygen consumption in both systems and blocks neither cleavage nor phosphorylation; (4) picric acid is completely inactive in both systems; (5) the concentrations of undissociated molecules required for each of five nitrophenols to block cleavage are exactly the same as those required to block phosphorylation.

That concentrations of 2,4-dinitrophenol ($5 \times 10^{-5}\ M$) which block phosphorylation in the cell-free system also block phosphorylation in the intact Arbacia egg was demonstrated by Abelson (1947) and by Villee, Lowens, Gordon, Leonard, and Rich (1949), who also found incorporation of P^{32} into all phosphate fractions to be inhibited; entrance of P^{32} into the DNA fraction being relatively most reduced.

Thus, there is reason to attribute the inhibition of cleavage to the interference with generation and transfer of "high energy" phosphate which the substituted phenols cause.

4. Naphthoquinones. The substance, 2-hydroxy-3(2-methyloctyl)-naphthoquinone-1,4 (SN 5949), prepared for test as an antimalarial, was found to have effects similar in some respects to those of the substituted phenols and in other respects to those of cyanide (Anfinsen, 1947). Below 7.4×10^{-8} *M*, respiration was stimulated; at 7.4×10^{-7} *M*, respiration of unfertilized and fertilized Arbacia eggs was inhibited by 50 per cent and 70 per cent, respectively; at the latter concentration cleavage was completely blocked.

Though the effects of this group of substances upon enzymes from eggs have not been studied, Ball, Anfinsen, and Cooper (1947) offer evidence that the naphthoquinones of this type may inhibit a respiratory step between cytochrome c and cytochrome b. It would be interesting to know whether echinochrome, the naphthoquinone pigment of the Arbacia egg, has similar effects.

5. Iodoacetate. Runnström (1935), using 0.03 *M* iodoacetate at pH values from 6.1 to 8.0, found that cleavage was abnormal and that the respiration of the fertilized eggs was strongly depressed; with maximal inhibition, the residual respiration is 40 per cent of the control rate. Addition of lactate or pyruvate plus the iodoacetate brings the respiration back nearly to normal, but the inhibition of cleavage is not overcome.

In sea water adjusted to pH 6.0 to permit penetration (see above), iodoacetic acid produces 50 per cent inhibition of cleavage at 1.7×10^{-3} *M* and 90 per cent inhibition at 3.4×10^{-3} *M*. At these concentrations, the rates of oxygen consumption are 67 and 54 per cent of the control values, respectively (Clowes and Krahl, 1940).

Ellis (1933) studied effects of iodoacetate and other reagents which combine with sulfhydryl groups upon cleavage of eggs of *Urechis caupo*.

Iodoacetate can combine with sulfhydryl groups and thereby inactivate enzymes which require this structure (Michaelis and Schubert, 1934; Hellerman, 1937; Barron and Singer, 1943). Its effects have not been studied on enzymes from the egg other than cytochrome oxidase, which it does not inhibit (Krahl, Keltch, Neubeck and Clowes, 1941).

6. Urethane and Related Carbamate Derivatives. Interest in this group of substances was initiated by the observations of Warburg (1910), who showed that 0.0005 *M* phenylurethane could block cleavage of Strongylocentrotus while reducing oxygen consumption by 20 per cent (at most). The effects of a number of other carbamate derivatives upon cleavage and oxygen consumption have now been studied (Table VIII). Cleavage in Arbacia was blocked by phenylurethane when oxygen consumption was reduced to 70 per cent of normal, the critical concentration being 0.001 *M* (Clowes and Krahl, 1940). Determinations of effects of carbamates upon cleavage of Arbacia, made prior to 1932, are summarized by Harvey (1932).

Urethane is one of the narcotic agents which Fisher and his co-workers have used in an effort to show whether or not the oxidative activities associated with

cleavage (activity respiration) proceed via pathways different from the respiration of the unfertilized egg (resting respiration). They analyzed mathematically the relation between narcotic concentration, respiration, and cleavage and concluded that the activity respiration passes over a catalytic system which is either different from that operating in the resting egg or has a different affinity for the inhibitor. Observations consistent with the same conclusions were made upon chloral hydrate, azide, and sulfanilamide (Fisher and Henry, 1944; Fisher, Henry, and Low, 1944).

Cornman (1947) determined effects of various carbamates on cleavage of Lytechinus.

The enzymatic mechanism underlying the specific effect of the carbamates on cleavage remains to be defined, but is obviously worthy of study.

7. Dyes. After the demonstration by Harrop and Barron (1928) that dyes capable of oxidation-reduction could enhance oxygen consumption of red cells, the effects of dyes upon Arbacia eggs were measured from a number of points of view: to determine whether dyes could substitute for oxygen as hydrogen acceptors in presence of respiratory inhibitors (Brooks, 1941); to determine whether the excess oxygen uptake could be used to accelerate cleavage and development (Clowes and Krahl, 1936; Brooks, 1941; Shapiro, 1948a); and as an aid in specifying the mechanism by which oxygen transfer is carried out in the eggs (Barron, 1929, 1932; Runnström, 1935; Korr, 1937; Brooks, 1941).

The individual investigators agree that, in concentrations from 10^{-4}–10^{-3} *M* at pH 7–8, methylene blue, pyocyanine, and a number of other dyes can increase oxygen consumption of both unfertilized and fertilized Arbacia eggs, but they do not agree completely in regard to the effects of the same agents upon cleavage and developmental processes. Clowes and Krahl (1936), studying cleavage over the three hour period after fertilization, found all dyes tested to produce irreversible cleavage block at the optimum concentration for respiratory stimulation. Shapiro (1948a), studying time required to reach first cleavage, likewise found an inhibition which, however, was reversible. The difference between the results of Shapiro and those of Clowes and Krahl may well lie in the length of time the eggs were exposed to the reagent. Brooks (1941, 1943), studying effects of KCN, CO, and methylene blue upon unfertilized eggs, and the early cleavage, morula, blastula, gastrula, and pluteus stages, found 0.0001 *M* methylene blue to increase the rate of development during early cleavage stages and to increase the size of the pluteus of Arbacia. Shapiro (1948a) could not confirm this favorable effect upon cleavage.

Brooks (1947) reported artificial parthenogenesis of eggs of *Urechis caupo* and *Strongylocentrotus purpuratus* by oxidation-reduction dyes.

8. Barbiturates and Local Anesthetics. The effects of 30 barbituric acid derivatives (Clowes, Keltch, and Krahl, 1940) and of 16 local anesthetics (Krahl, Keltch, and Clowes, 1940a) upon cleavage of the Arbacia egg were determined in the course of a study of their mechanism of penetration into living cells. The results are given in Tables VI and VII.

With a representative barbiturate, 5-isoamyl-5-ethyl barbituric acid, the oxygen consumption was 75 per cent of control when division was inhibited 50 per cent and 20 per cent of control when division was inhibited completely. A typical local

anesthetic, γ-(2-methylpiperidyl)-propyl benzoate, produced 64 per cent inhibition of cleavage while oxygen consumption remained at, or slightly above, normal. Thus, the local anesthetic bases, like azide, sulfide, and the carbamates, have specific inhibitory effects upon cleavage which are not dependent upon inhibition of oxygen consumption.

The actions of barbiturates and local anesthetics upon enzyme systems of the Arbacia egg have not been studied.

TABLE VI

Concentrations of substituted barbituric acids required to produce 50 per cent reduction of the rate of cell division of fertilized eggs of Arbacia punctulata at 20° C. and approximately pH 7.8–8.0 [1]

(From Clowes, Keltch, and Krahl, 1940)

Substituted barbituric acid	pK′ at 20° for sea water	Final pH of medium	Total concentration for 50 per cent inhibition of cleavage
			moles per l. × 10^3
1. 5-ethyl-5-(β-ethylhexyl)	7.75	8.00	2
2. 5-allyl-5-(α-methylbutyl)	7.92	7.81	2
3. 5-benzyl-5-isopropyl	7.82	7.97	4
4. 5-(β-bromoallyl)-5-(α-ethylpropyl)	7.58	8.00	5
5. 5-ethyl-5-(α-methylbutenyl-2)	7.78	7.99	5
6. 5-(β-methylallyl)-5-(α-methylbutyl)	7.80	7.86	7
7. 5-ethyl-5-hexyl	7.66	8.00	8
8. 5-ethyl-5-(α-methylbutyl)	8.02	8.00	12
9. 5-(β-bromoallyl)-5-(α-methylpropyl)	7.60	8.00	13
10. 5-ethyl-5-(α-ethylpropyl)	7.89	7.98	14
11. 5-allyl-5-benzyl	7.06	7.92	15
12. 5-amyl-5-ethyl	7.82	7.93	15
13. 5-(α, γ-dimethylbutyl)-5-ethyl	7.99	7.90	15
14. 5-isoamyl-5-ethyl	7.78	8.04	15
15. 5-cyclopentenyl-5-ethyl	7.98	7.97	17
16. 5-ethyl-5-(β-phenylethyl)	7.74	7.99	22
17. 5-allyl-5-isobutyl	7.54	8.00	39
18. 5-isobutyl-5-(β-methylallyl)	7.62	7.93	40
19. 5-butyl-5-ethyl	7.76	7.97	40
20. 5-sec-butyl-5-crotyl [2]	—	8.03	42
21. 5-(β-bromoallyl)-5-isopropyl	7.59	8.00	48
22. 5-ethyl-5-cyclohexenyl	7.36	8.00	58
23. 5-ethyl-5-phenyl	7.26	7.92	95
24. 5-allyl-5-isopropyl	7.73	7.97	98
25. 5-cyclohexenyl-1,5-dimethyl	8.24	8.74	104
26. 5,5-diallyl	7.62	7.96	160
27. 5-ethyl-5-isopropyl	7.90	8.02	162
28. 5-ethyl-5-piperidyl	7.56	7.92	200
29. 5,5-diethyl	7.75	7.94	320

[1] The concentrations required at pH 6.4–6.7 and at pH 8.6–8.9 were also determined.

[2] Derivative of thiobarbituric acid rather than of barbituric acid itself.

9. MISCELLANEOUS. The effects of many other agents, with unknown effects upon metabolic enzymes, have been tested upon respiration and cleavage of Arbacia eggs.

Since these do not contribute directly to the principal objective of this paper, namely, to elucidate the metabolic processes by which energy is provided by cleavage, they will not be dealt with here in detail. But all the miscellaneous agents which have been employed in the period 1932–1949 are listed below, and quantitative data for them, where available, are given in Tables VIII and IX.

TABLE VII

Concentrations of local anesthetics required to produce 50 per cent reduction of the rate of cell division of fertilized eggs of Arbacia punctulata at 20° C. and approximately pH 7.8–8.0[1]

(From Krahl, Keltch, and Clowes, 1940)

Local anesthetic	pK′ at 20° for sea water	Final pH of medium	Total concentration for 50 per cent inhibition of cleavage
			moles per l. × 10^4
1. N,N-Diethyl-N′-(2-butyloxy-quinoline-4-carboxyl)-ethylenediamine ('Dibucaine')	5.03	7.99	0.06
2. α-Piperidyl-β,γ-dicarbanilido-propanediol ('Diothane')	5.84	7.94	0.3
3. β-Dimethylaminoethyl-p-butylaminobenzoate ('Tetracaine')	5.50	7.98	0.9
4. 4,4′-Diethoxy-N,N′-diphenyl-amidine ('Phenacaine')	4.78	7.90	1.3
5. 2-Benzoxy-2-methyl-1-dimethylaminobutane ('Stovaine')	5.42	7.90	1.3
6. γ-Dibutylaminopropyl-p-aminobenzoate ('Butacaine')	4.90	7.91	3.1
7. 4-Benzoxy-2,2,6-trimethylpiperidine ('β-Eucaine')	4.27	7.90	4.5
8. γ-Diethylaminopropylcinnamate ('Apothesine')	4.06	7.93	5.2
9. β-Diethylaminoisohexyl-p-aminobenzoate ('Panthesine')	4.44	7.90	7.0
10. 2-Benzoxy-2-dimethylaminomethyl-1-dimethylaminobutane ('Alypin')	4.55	7.92	7.2
11. N-Methyl-l-benzoylecgonine ('Cocaine')	4.97	7.78	10
12. β,β-Dimethyl-γ-diethylamino-propylbenzoate ('Larocaine')	4.21	7.91	10
13. γ-(2-Methylpiperidyl)-propylbenzoate ('Metycaine')	4.18	8.00	13
14. Ethyl-p-aminobenzoate ('Benzocaine')	10.82	7.97	15
15. β-Diethylaminoethyl-p-aminobenzoate ('Procaine')	4.73	7.93	23
16. 2-(p-Aminobenzoxy)-2-methyl-1-dimethylaminobutane ('Tutocaine')	4.82	7.93	45

[1] The concentrations required at pH 6.4–6.7 and at pH 8.6–8.9 were also determined.

Acetyl salicylic acid—Budington (1935)

Acids and bases—Clowes, Keltch, and Wade (1933, 1934); Keltch, Wade, and Clowes (1933)

Amyl alcohol—Clowes and Keltch (1941)

Caffeine and related compounds—Cheney (1945, 1948)

TABLE VIII

Effect of various substances upon oxygen consumption and cleavage of fertilized Arbacia eggs

Agent	T° C.	pH	Oxygen consumption			Cleavage		
			Maximal change produced	Conc. required to produce maximal change	Change at conc. required to inhibit cleavage maximally	Maximal inhibition	Conc. required to produce maximal inhibition	Reversibility of maximum inhibition
			per cent of control	moles per l. × 10^4	per cent of control	per cent of control	moles per l. × 10^4	
Miscellaneous								
n-Amylalcohol [1]	20	8.0				100	660	none
Caffeine [2]	25	7.9	−60	250	−60	100	250	not stated
Carbon dioxide [3]	21		−80		−70	—	—	not stated
Chloral hydrate [6]	24	7.3–8.3	−80	1000	−60	100	63	partial
Chloroform [1]	20	8.0				100	160	none
Colchicine [4]						100	1	not stated
Colchicine [6]						100	2	not stated
Ethyl carbamate [6]	24	7.3–8.3	−75	6300	−30	100	1400	yes
Hydroxyethylapocupreine [1]	20	7.8				100	41	none
p-(α-Methylbutylamino) benzoate [1]	20	7.8				100	10	partial
Penicillin [7] (non-crystalline)	20	7.5				100	5000 [7]	not stated
Phenylmercury nitrate [1]	20	6.9–8.6				100	0.02	none
Podophyllotoxin [8]						100	0.01	not stated
Tetrachlorohydroquinone [1]	20	7.9				100	0.6	none
Tetrachloroquinone [1]	20	7.9				100	1	none
Usnic acid [9]						100	3	not stated
Sulfonamides and related compounds								
n-Butylsulfone-p-phenetidide [1]	20	7.9				65	20	yes
Diaminodiphenylmethane [1]	20	7.9				100	50	yes
Diaminodiphenylsulfone [1]	20	7.9	−43	20	−35	60	9	yes
Ethylsulfone-p-phenetidide [1]	20	7.9				100	16	yes
Methylsulfone-p-phenetidide [1]	20	7.9				40	41	yes
p-Nitrosobenzenesulfonamide [1]	20	7.9			0	100	2	none
n-Propylsulfone-p-toluidide	20	7.9				100	20	yes
Sulfanilamide [10]	25	8.0	−48	400	−45	100	400	yes
Sulfanilylethanolamine [1]	20	7.9				60	280	none
N^4-Trichloroacetyl-sulfanilamide [1]	20	7.9				70	20	none

TABLE VIII—*Continued*

Agent	T° C.	pH	Oxygen consumption			Cleavage		
			Maximal change produced	Conc. required to produce maximal change	Change at conc. required to inhibit cleavage maximally	Maximal inhibition	Conc. required to produce maximal inhibition	Reversibility of maximum inhibition
			per cent of control	moles per l. × 10^4	per cent of control	per cent of control	moles per l. × 10^4	
Dyes								
o-Cresol indophenol [12]	20	7.9	+75	3	+30	100	1	none
Dimethyl-p-phenylene diamine [12]	20	7.9	+125	10	+120	100	2	none
Methylene blue [12]	20	7.9	+60	10	+50	100	5	none
Neutral red [12]	20	7.9	+25	1	0	90	10	none
Pyocyanine [12]	20	7.9	+200	5	+90	100	30	none
Pyocyanine [11]	—	—	+96	5	—			
Tetramethyl-p-phenylene diamine [12]	20	7.9	+250	10	+250	100	10	none
Dithiocarbamates								
sec-Butyl ethyldithiocarbamate [1]	20	7.9				80[13]	0.3	none
Di-n-butyldithiocarbamate [1]	20	7.9				100	41	none
Diethyldithiocarbamate [1]	20	7.9	+40	1	0	85[13]	0.3	none
Di-isoamyldithiocarbamate [1]	20	7.9				100	5	none
Dimethyldithiocarbamate [1]	20	7.9				50	81	none
Di-n-propyldithiocarbamate [1]	20	7.9				100	0.3	none
N,N′-Dimethyl-(2,4-dinitrophenyl)-dithiocarbamate [1]	20	7.9				100	20	none
Isoamylethyldithiocarbamate [1]	20	7.9				100[13]	0.2	none
Isobutylethyldithiocarbamate [1]	20	7.9				100[13]	0.3	none

[1] Clowes and Keltch (1939, 1940, 1941). [2] Cheney (1945, 1948).

[3] Root (1930). The maximal effect on oxygen consumption was measured at 180 mm. CO_2, 0.0018 *M* $NaHCO_3$, pH 5.50. The maximal effect on cleavage as measured by the cleavage delay method was obtained at 40 mm. CO_2, 0.0018 *M* $NaHCO_3$, pH 6.15, at which point oxygen consumption was reduced by 70 per cent.

[4] Nebel (1937). [5] Beams and Evans (1939). [6] Fisher and Henry (1944).

[7] Henry and Henry (1945). The concentration is stated in Oxford units per ml.

[8] Cornman (1947). [9] Marshak and Harting (1948). [10] Fisher, Henry, Low (1944).

[11] Runnström (1935b). Pyocyanine at 5 × 10^{-4} *M* also increased oxygen consumption of unfertilized eggs by 210 per cent.

[12] Clowes and Krahl (1936).

[13] A smaller degree of inhibition was produced by higher concentrations of this substance. This effect, while reproducible, is not understood.

TABLE IX

Delay in first cleavage of fertilized Arbacia eggs produced by various chemical agents

Agent	Concentration	T° C.	pH	Delay in first cleavage
				minutes
CO_2-bicarbonate [1]	10 mm. CO_2-0.0018 *M* $NaHCO_3$	20	6.76	16
	40 mm. CO_2-0.0073 *M* $NaHCO_3$		6.70	44
	80 mm. CO_2-0.0143 *M* $NaHCO_3$		6.67	108
	20 mm. CO_2-0.0018 *M* $NaHCO_3$		6.41	30
	80 mm. CO_2-0.0073 *M* $NaHCO_3$		6.41	260
Methylene blue [2]	1×10^{-4} *M*	26		2
	2×10^{-4} *M*			5
	4×10^{-4} *M*			10
Toluylene blue [2]	4×10^{-4} *M*	26		57
Ascorbic acid [3]	4×10^{-5} *M*	26.1		6
	2×10^{-4} *M*			7
	9×10^{-4} *M*			16
	2×10^{-3} *M*			30

[1] Haywood and Root (1932).
[2] Shapiro (1948a).
[3] Shapiro (1948b).

Carbon dioxide—Smith and Clowes (1924); Root (1930); Haywood and Root (1930, 1932)

Carcinogenic hydrocarbons—Keltch, Krahl, and Clowes (1937)

Chloral hydrate—Fisher and Henry (1944)

Chloroform—Clowes and Keltch (1941)

Colchicine—This substance is said to stop mitosis at metaphase, Nebel (1937); Beams and Evans (1940)

Dithiocarbamates—Clowes and Keltch (1941)

Ethyl alcohol—Waterman (1936)

Hydroxyethylapocupreine—Clowes and Keltch (1940)

Hypotonic sea water—Cornman (1943)

Leukotaxine—Menkin (1940)

Malonic acid—Rulon (1948)

Malononitrile—This substance interferes with phosphate transfer in intact eggs, Villee, Lowens, Gordon, Leonard and Rich (1949)

Metal ions—Alkali metals (Li, Na, K)—Chambers and Chambers (1938); Runnström (1935c); Moore, Bliss, and Anderson (1945); *copper*—Glaser (1924); Finkel, Allee, and Garner (1942); Runnström (1939); *other heavy metals*—Page (1929); Waterman (1937)

Nitrogen mustards—Agents of this group have been studied by Barron, Seegmiller, Mendes, and Narahara (1948) and by Hutchens and Podolsky (1948). No inhibition of oxygen consumption of the eggs was observed. Hutchens and Podolsky found the cleavage over a 3 hour period to be reduced to 75 per cent of control

by tris-(β-chloroethyl)amine at 3×10^{-5} *M* and by various bis-(β-chloroethyl) amines at 2×10^{-4} *M*.

Penicillins—Non-crystalline samples of penicillin had a slight inhibitory effect on cleavage at concentrations several thousand times those required to stop growth of sensitive bacteria (Henry and Henry, 1945), but highly purified samples of various penicillins were virtually without effect upon either respiration or cleavage (Clowes and Keltch, 1946).

Phenylmercury nitrate—Clowes and Keltch (1940)

Podophyllotoxin—Corriman (1947)

Pressure—Marsland (1939)

Rhodamine B—Clark (1940)

Sulfhydryl-blocking agents—Barron, Nelson, and Ardao (1948); Clowes and Keltch (1940)

Sulfonamides—Fisher, Henry, and Low (1944); Clowes and Keltch (1939)

Temperature—Rubenstein and Gerard (1933); Hoadley and Brill (1937); Tyler and Humason (1937); Borei (1949); Borei and Lybing (1949)

Tetrachlorohydroquinone—Clowes and Keltch (1940)

Ultraviolet light—Giese (1938a, 1938b, 1939); Harvey and Hollaender (1938)

Uranyl nitrate—Effects of this substance upon egg respiration have not been reported, but it inhibits respiration of Arbacia sperm (Benedict and Barron, 1946). It also inhibits phosphate transfer in the intact egg (Villee, Lowens, Gordon, Leonard, and Rich, 1949).

Usnic acid—This substance inhibits cleavage and phosphate transfer (Marshak and Harting, 1948), and also desoxyribonuclease (Marshak, 1949).

Vitamin C—Shapiro (1948b)

X-rays—Henshaw (1932, 1938, 1939); Henshaw and Francis (1936); Cohen (1939); Evans (1940); Evans and Beams (1939)

The following agents have been found to be without effect upon cleavage of fertilized Arbacia eggs when tested at the molar concentrations shown, which were the highest concentrations employed (Clowes and Keltch, 1939–1941): diethylmethylcarbinylurea, 8×10^{-3}; 4,4′-dinitrobenzophenone, 6×10^{-4}; 2,4-dinitrophenylmercaptobenzothiazole, 2×10^{-3}; morphine, 2×10^{-2}; phenacetine, 8×10^{-3}; porphyrexide, 1×10^{-3}; 2-sulfanilylaminothiazole, 5×10^{-4}; N-sulfanilylbenzamide, 5×10^{-4}; sulfapyridine, 8×10^{-3}; synthalin, 2×10^{-3}.

G. Summary

1. This review deals with the general question of relation of metabolic activities to cleavage in the Arbacia egg. According to current biochemical concepts the answer to this question would be found in complete information about the following subjects:

a. The identity and interrelationships of the catalysts by which the eggs derive energy from foodstuffs.

b. The reactions by which energy is transferred to make possible the synthesis of structural and functional elements of the egg, such as carbohydrates, proteins, lipids, coenzymes, nucleic acids and other substances, many of which may yet be unknown.

c. The chemical and physical chemical rearrangements of these structural functional elements which lead to the biological event of cleavage.

The third subject is completely beyond investigation by present techniques, and the second is just being opened to investigation in the living egg by application of isotopic labelling techniques. This review is concerned, therefore, chiefly with the nature and interrelationships of the catalysts by which Arbacia eggs derive energy from foodstuffs.

2. The metabolic catalysts which have been shown to be *available* in the Arbacia egg for deriving energy from foodstuffs are:

a. An enzyme with some, but not all, of the properties of the cytochrome oxidase of other animal tissues. After extraction from the egg it oxidizes cytochrome c, but whether this activity is related to its biological function in the egg is an open question, as no biochemically significant quantity of cytochrome c has yet been conclusively demonstrated in the eggs, although it is present in the sperm.

The enzyme is poisoned by carbon monoxide, cyanide, azide, and sulfide, but not by copper-binding reagents. Its relative affinities for oxygen and carbon monoxide are, in the cell-free state, the same as those for cytochrome oxidase of mammalian muscle but, in the intact Arbacia egg, these relative affinities are intermediate between those of the usual cytochrome oxidase and those of other iron-porphyrin compounds such as hemoglobin.

b. The coenzymes, flavin-adenine dinucleotide, diphosphopyridine nucleotide, and diphosphothiamine. The properties of the enzymes of which these coenzymes are parts have not been investigated.

c. A mechanism for oxidative disposal of pyruvate.

d. An enzyme complex which can oxidize oxalacetate, α-ketoglutarate, or succinate with concomitant disappearance of inorganic phosphate, presumably via generation of "high energy" phosphate bonds and esterification of suitable phosphate acceptors.

e. The following enzymes which have undefined roles with respect to energy production and to chemical syntheses in the egg: dehydrogenases for various substrates other than those already mentioned; phosphatases; ribonuclease; desoxyribonuclease; catalase; peptidases.

The distribution of various enzymes in the egg has been studied with very suggestive findings. In the unfertilized or recently fertilized egg the total reducing activity, cytochrome oxidase, and desoxyribonuclease are quantitatively most abundant in the cytoplasmic matrix, i.e. in solution or upon the small granules less than 1 μ in diameter. As development proceeds, a progressively greater fraction of the enzymes are associated with larger granules of the type which stain as mitochondria. In view of these observations and the fact that the capacity for oxidative phosphorylation is found, in the adult mammalian tissues so far studied, to be associated with mitochondrial particles, the suggestion arises that localization of enzymes on specific particles may parallel development of specific function.

3. What is the *actual* pathway connecting enzyme activities with cleavage? Such answers to this question as are available come largely from experiments designed to block a specific metabolic enzyme system and to observe the degree to

which the egg can or cannot cleave when deprived of this enzyme or group of enzymes.

A note of caution should be emphasized here. The problem of explaining physiological effects in chemical terms is an exceedingly difficult one. It involves the demonstration not only that a given effect of a reagent upon an enzyme is *sufficient* to explain the physiological effect, but also the demonstration that the chemical effect offered as explanation is the only one possible, i.e. is a *necessary* explanation. Only the sufficiency criterion is usually met. In view of these considerations it is clear that conclusions about the relation of enzymes to function, which are based on action of inhibitors, must be only provisional. With this reservation in mind, the main findings about enzymatic activities and cleavage may be summarized.

a. For energy to support cleavage, eggs of Arbacia are completely dependent on aerobic processes. Like other animal cells, the eggs can form lactic acid but this process cannot support cleavage.

The nature of the foodstuffs oxidized is not settled; evidence for oxidation of carbohydrate or its phosphorylated breakdown products, of fats, or of amino acids has been offered, but is not conclusive.

b. Evidence for participation of two enzyme systems in oxidative energy-yielding reactions has been presented.

(1) *The cytochrome oxidase system.* The principal indication for participation of this oxidative step is that both the inhibition of oxygen consumption and the inhibition of cleavage by carbon monoxide are *light reversible.* Cytochrome oxidase is the only metabolic catalyst known to be subject to light-reversible inhibition by carbon monoxide.

(2) *An oxidative phosphorylation system.* The principal evidence is the highly specific parallel between inhibition of cleavage and inhibition of a cell-free phosphorylating system from the eggs by a series of substituted phenols. Supporting evidence is found in the fact that these agents likewise block phosphorylation and phosphate transfer in the intact fertilized egg.

c. In an effort to reveal the metabolic steps essential for cleavage, comparisons of the metabolic activities of unfertilized and fertilized eggs have been made.

The oxygen consumption of intact Arbacia eggs increases upon fertilization to a degree which depends on the temperature, by a factor of about 10 times at 11° C., by a factor of about 2 times at 30° C., and negligibly at 32° C. The higher respiration of the fertilized egg is relatively more sensitive than that of the unfertilized both to inhibitors of cytochrome oxidase, such as cyanide and carbon monoxide, and to inhibitors which act at other loci, such as narcotics.

The concentration of cytochrome oxidase is the same in unfertilized and fertilized eggs, and in great excess relative to oxygen consumption of intact eggs. The concentration of total dehydrogenase activity, as measured by ability to reduce ferricyanide, is likewise unchanged upon fertilization.

The ability to dispose of pyruvate, on the other hand, appears to be increased following fertilization.

From these and related facts, all observers appear to agree that the increase in oxygen consumption upon fertilization is the consequence of the introduction of a new or accelerated step between some substrate, perhaps pyruvate, and cytochrome

oxidase. The nature of the new or accelerated step, a frequent subject of speculation, is unknown. It is not known to what extent, if any, the energy liberated by the excess oxygen consumption is used for synthetic processes and for cleavage.

The precise mechanism by which oxidative energy is made available for cleavage remains to be defined.

4. In the interest of completeness, and to provide a foundation for the further study of relation of metabolic activities and cleavage, the effects of all agents so far tested upon oxygen consumption and cleavage of Arbacia eggs have been recorded, even though their effects upon specific enzymatic activities frequently still await investigation. Examples of the viewpoints from which these substances have been investigated, and of the agents employed, are:

a. To demonstrate participation of various enzyme systems in yielding energy for cleavage: poisons for cytochrome oxidase such as cyanide, carbon monoxide, azide, sulfide; poisons for phosphorylation processes such as substituted phenols, azide, usnic acid; and poisons for other metabolic enzymes such as naphthoquinones and iodoacetate.

b. To learn more about the mechanisms by which physiologically active substances affect growth and cell division: acids and bases, carbamates, colchicine, nitrogen mustards, sulfonamides, penicillin.

c. To determine the form in which toxic ionizable substances penetrate living cells such as Arbacia eggs: substituted phenols, barbiturates, local anesthetics, other weak acids and bases. These agents appear to gain access to the interior of the Arbacia egg chiefly, if not entirely, as undissociated molecules.

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